PATENT
Docket No. 432722002623

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Marian Christopher

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Gregory R. MUNDY, et al.

Serial No.: 09/695,807

Filing Date: October 23, 2000

For: INHIBITORS OF PROTEASOMAL ACTIVITY FOR STIMULATING BONE GROWTH

Examiner: R. Gitomer

Group Art Unit: 1651

DECLARATION OF I. ROSS GARRETT PURSUANT TO 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, I. Ross Garrett, declare as follows:

1. I am one of the co-inventors of the above-referenced patent application, and am familiar with the contents thereof.

2. Other co-inventors and I have conducted experiments demonstrating that proteasome inhibitors promote osteoblast proliferation, differentiation of osteoblast precursors and bone growth. These experimental results are set forth in the following paragraphs 3-10 and in the attached Tables 1-6 and Figures 1-2.

Exhibit D

3. Osteoblast proliferation, differentiation of osteoblast precursors and bone growth can be shown by histologic observations of calvarial bone cultures, which show increased osteoblast numbers, BMP induction, new bone formation and morphologic maturation of osteoblasts. Detailed disclosure of methods for determining new bone area and osteoblast numbers is in the specification at page 20, line 11 to page 21, line 11 and in Example 2 at page 35, line 16 through page 36, line 26.

4. Compounds that stimulate bone growth can be shown by analyzing the induction of BMP mRNA and protein. Disclosure of these methods for identification of bone anabolic agents is found in the specification at page 3, lines 24-25, page 19, lines 7-11, and page 20, lines 5-6. Analysis of mRNA and protein expression can be performed using assays well-known in the art.

5. Osteoblast proliferation and bone growth can be shown by histomorphometric analysis of bone following *in vivo* treatment with a compound of interest. Detailed disclosure of methods for morphometric analysis is found in the specification at page 21, line 12 to page 24, line 9, Examples 3 and 4 at page 37 to page 39, and Example 7, at page 41.

6. Promotion of osteoblast proliferation and new bone growth by structurally-unrelated proteasome inhibitors is demonstrated in the Table 1 submitted herewith. A number of these compounds were tested for their capacity to stimulate bone formation in calvarial organ cultures and inhibit proteasomal activity assay as described in Paragraph 3 *supra*. The ten structurally unrelated proteasomal inhibitors examined included epoxomicin, AcLL, eponemycin, lactacystin, YU101, AcNapthLLL, PSI, MG132, MG115, and ALLN. Data are expressed as ED_{50}, the dose required to elicit half of the maximal response for either bone formation or proteasomal inhibitory activity. The ability of the compounds to inhibit proteasomal activity was determined using proteasome activity as described in specification at Example 5. The ability to stimulate bone formation was determined using the *in vitro* bone formation assay exemplified in Example 2. Specifically, explants of neonatal murine calvarial bone were cultured for 72 hours in the presence of the compound and then examined histomorphometrically. A strong positive correlation (R^2=0.94) was demonstrated between the capacity of each of these compounds to inhibit proteasomal activity and their bone forming activity. The most potent and selective inhibitor of the chymotrypsin-like site, the amino-

terminally acetylated epoxyketone peptide YU101, stimulated bone formation at concentrations of 10 nM.

7. The specificity of proteasome inhibitors on BMP-2 expression was determined by examining the effects of the proteasome inhibitors PSI on mRNA expression of BMP-2, BMP-4, BMP-6 in fetal calvarial cells (FRC) osteoblasts *in vitro* as described in Paragraph 4 *supra*. The proteasomal inhibitors PSI increased BMP-2 mRNA expression, but had no significant effects on mRNA expression of BMP-4 and BMP-6 as shown in Figure 1.

8. Proteasomal inhibitors were examined for their capacity to increase accumulation of BMP2 protein in conditioned media from human Hu09 osteoblastic cells by coculturing the cells with the inhibitors at multiple concentrations for 24 hours as shown in Table 2. Protein levels in the media were determined using a commercially available ELISA kit for BMP2 (Quantikine, R&D Systems). The structurally-unrelated proteasomal inhibitors tested were YU101, proteasome inhibitor-1, epoxomicin, MG132, MG115, and lactacystin. Data are expressed as mean $\pm$SEM where * designates $p<0.05$ versus vehicle alone. All of the proteasomal inhibitors tested induced statistically significant increases in the BMP-2 protein production from osteoblastic cells when compared to cells treated with vehicle alone.

9. The inhibitory ability of structurally-unrelated proteasomal inhibitors positively correlated with the ability to stimulate the expression of BMP-2 protein in Hu09 osteoblastic cells as shown in Table 3. The proteasomal inhibitors epoxomicin, lactacystin, YU101, PSI, MG132, and MG115 were analyzed as described in Paragraph 4 *supra*. Data are expressed as the doses expressed as ED_{50} are the doses required to elicit half of the maximal response for BMP2 protein production and proteasomal activity inhibition. In every case, the ability to inhibit proteasomal activity correlated with the induction of BMP-2 expression (R^2=0.95).

10. The ability of structurally-unrelated proteasomal inhibitors to stimulate bone formation positively correlated with the ability to stimulate the expression of BMP-2 protein in Hu09 osteoblastic cells as shown in Table 4. The proteasomal inhibitors epoxomicin, lactacystin, YU101, PSI, MG132, and MG115 were analyzed as described in Paragraphs 3 and 4 *supra*. Data are expressed as the doses expressed as ED_{50} are the doses required to elicit half of the maximal response for either BMP2 protein production and bone formation. In every case, the ability to stimulate bone formation correlated with the induction of BMP-2 expression (R^2=0.95).

12. The ability of proteasome inhibitors to stimulate new periosteal bone formation in the calvaria of mice was determined as described in Paragraph 5 *supra*. To perform these experiments, compounds were injected into the subcutaneous tissue over the calvaria of normal mice. 5-weeks old Swiss ICR white mice were injected 3 times/day for 5 days with either vehicle alone, epoxomicin or PSI over the right side of the calvarium. Mice were euthanized on day 22 and calvaria removed for histomorphometric analysis. Data are expressed as mean +/- SEM where * designates $p<0.001$ versus treatment with vehicle alone. Both PSI and epoxomicin stimulated new bone formation in a dose-dependent manner and did not cause toxic effects in this dose range as shown in Table 5.

13. *In vivo* administration of proteasomal inhibitors also demonstrated the ability of these inhibitors to stimulate bone formation using the methods as described in Paragraph 5 *supra*. Briefly, histologic sections, bone formation rates and mineral apposition rates of murine proximal tibia were determined from mice treated daily for 5 days with (a) vehicle alone, (b) PTH at 0.08 mg/kg/day subcutaneously, (c) PSI at 2 mg/kg/day intraperitoneally, (d) epoxomicin at 0.1 mg/kg/day intraperitoneally. The mice were subsequently sacrificed 16 days later for bone analysis. In Figure 2, values in parentheses are percent change from vehicle-treated controls. BV/TV bone volume/tissue volume; BFR, bone formation rate; MAR mineral apposition rate. PSI was administered subcutaneously and epoxomicin was administered intraperitoneally to normal intact Swiss white mice for 5 days. Parathyroid hormone (PTH) was used as a control, since when used over longer periods it has powerful anabolic effects on bone formation. However, PTH did not have significant effects with only 5 days of daily subcutaneous administration (Figure 2b). PSI caused an increase in trabecular bone volume of 28 percent accompanied by a 71 percent increase in bone formation rates measured by dynamic parameters compared with vehicle-treated mice (Figure 2c). This increase in bone formation rate was present when the animals were sacrificed, which was over two weeks after the compound was last administered. This increased bone formation rate shows the increase in trabecular bone volume was due to an absolute increase in the rate of bone formation rather than a secondary effect due to a decreased bone resorption. There were no effects of these compounds on rates of bone resorption in organ cultures and the measured increases in rates of bone formation are so pronounced that the increases in bone volume cannot be ascribed to a simple reduction in bone

turnover. Epoxomicin (Figure 2d) caused similar responses to those of PSI, but at doses at least 20-fold less.

14. The effects of proteasome inhibitors on trabecular bone volume in mice was determined as described in Paragraph 5 *supra*. PSI was injected subcutaneously, and epoxomicin was injected intraperitoneally in normal intact Swiss white mice. PSI was injected into mice at 1-2 mg/kg/day for 5 days. Epoxomicin was injected into mice at doses between 0.5-0.004 mg/kg/day for 5 days . The data shown in Table 6 demonstrate that treatment with epoxomicin and PSI caused a significant increase in bone formation in a dose responsive manner. Data were expressed as mean +/- SEM where * designates $p<0.001$ versus treatment with vehicle alone. At the highest dose of epoxomicin, a 98% increase in the bone volume/tissue volume. The increases in bone formation rates and bone volumes after only 5 days of treatment are of comparable degree to those that require over one month of treatment with other anabolic agents such as the statins and PTH.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

4-22-2003
Date

I. Ross Garrett

TABLE 1

Compound	Bone Formation Activity (nM) ED_{50}	Proteasome Inhibitory Activity (nM) ED_{50}
Epoxomicin	10	100
AcLLL	3000	3000
Eponemycin	250	625
Lactacystin	2000	1000
YU101	10	60
AcNapthLLL	40	200
PSI	32	100
MG132	500	625
MG115	1000	1000
ALLN	5000	1600

TABLE 2

Treatment	Concentration (uM)	BMP2 protein (pg/ml)
YU101	Vehicle alone	37 ± 5
	0.0025	56 ± 15
	0.005	59 ± 20
	0.010	119 ± 22*
	0.020	163 ± 12*
	0.040	212 ± 27*
Proteasome inhibitor-1	Vehicle alone	67.2 ± 5
	0.0062	98 ± 12*
	0.0125	139 ± 15*
	0.025	158 ± 18*
	0.050	152 ± 22*
	0.100	156 ± 16*
Epoxomicin	Vehicle alone	36 ± 6
	0.01	50 ± 8
	0.02	108 ± 14*
	0.04	172 ± 9*
	0.08	75 ± 11*
MG132	Vehicle alone	71 ± 13
	0.035	65 ± 4
	0.075	84 ± 13
	0.100	107 ± 11*
	0.300	140 ± 14*
	0.600	145 ± 6*
MG115	Vehicle alone	71 ± 12
	0.300	71 ± 11
	0.600	149 ± 15*
	0.1200	74 ± 3
Lactacystin	Vehicle alone	36 ± 6
	125	37 ± 8
	250	83 ± 12*
	500	117 ± 15*

TABLE 3

Compound	Proteasome Inhibitory Activity (nM) ED_{50}	BMP2 expression (nM) ED_{50}
Epoxomicin	100	10
Lactacystin	1000	300
YU101	60	15
PSI	100	20
MG132	625	150
MG115	1000	300

TABLE 4

Compound	Bone Formation Activity (nM) ED_{50}	BMP2 expression (nM) ED_{50}
Epoxomicin	10	10
Lactacystin	2000	300
YU101	10	15
PSI	32	20
MG132	500	150
MG115	1000	300

TABLE 5

Treatment	Dose (mg/kg/day)	Total Bone Area (um^2)	% Increase
PS1	Vehicle alone	0.64 ± 0.03	
	0.1	0.74 ± 0.02*	22
	1	0.83 ± 0.02*	35
	5	0.79 ± 0.03*	32
Epoxomicin	Vehicle alone	0.68± 0.02	
	0.05	0.78±.03*	15
	0.1	0.87±.02*	28
	0.5	0.91±.03*	34

TABLE 6

Treatment	Dose (mg/kg/day)	% BV/TV	% Increase
PS1	Vehicle alone	13.1 ± 2.2	
	1	22.3 ± 2.4*	36
	2	17.6 ± 1.2*	25
Epoxomicin	Vehicle alone	11.7 ± 3.4	
	0.004	14.3 ± 1.8	22
	0.02	18.5 ± 1.0*	58
	0.1	23.2 ± 4.2*	98

FIGURE 1

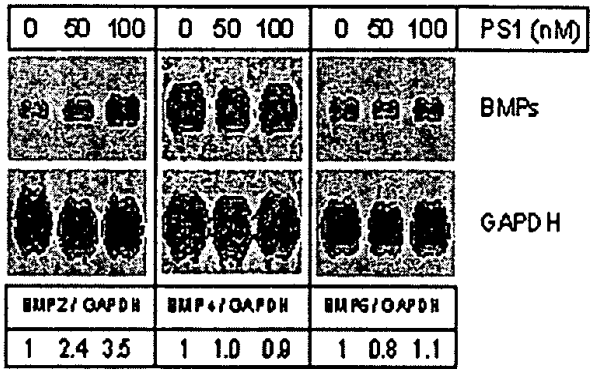
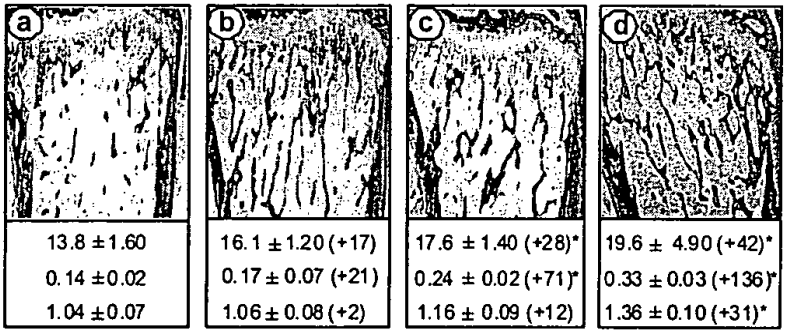


FIGURE 2



	a	b	c	d
BV/TV	13.8 ±1.60	16.1 ±1.20 (+17)	17.6 ± 1.40 (+28)*	19.6 ± 4.90 (+42)*
BFR	0.14 ±0.02	0.17 ± 0.07 (+21)	0.24 ± 0.02 (+71)*	0.33 ± 0.03 (+136)*
MAR	1.04 ±0.07	1.06 ± 0.08 (+2)	1.16 ± 0.09 (+12)	1.36 ± 0.10 (+31)*

PTO/SB/25 (10-00)
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TERMINAL DISCLAIMER TO OBVIATE A PROVISIONAL DOUBLE PATENTING REJECTION OVER A PENDING SECOND APPLICATION	Docket Number (optional) 43272200262

In re Application of: Gregory R. MUNDY, et al.

Application No.: 09/421,545

Filed: October 20, 1999

For: INHIBITORS OF PROTEASOMAL ACTIVITY AND PRODUCTION FOR STIMULATING BONE GROWTH

The owner, Osteoscreen, Inc., of one hundred percent [100%] interest in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application, which would extend beyond the expiration date of the full statutory term defined in 35 U.S.C. 154 to 156 and 173 as presently shortened by any terminal disclaimer, of prior Patent No. 6,462,019. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and the prior patent are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns.

In making the above disclaimer, the owner does not disclaim the terminal part of any patent granted on the instant application that would extend to the expiration date of the full statutory term as defined in 35 U.S.C. 154 to 156 and 173 of any patent granted on the second application, as shortened by any terminal disclaimer filed prior to the patent grant, in the event that any such granted patent: expires for failure to pay a maintenance fee, is held unenforceable, is found invalid by a court of competent jurisdiction, is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321, has all claims canceled by a reexamination certificate, is reissued, or is in any manner terminated prior to the expiration of its full statutory term as shortened by any terminal disclaimer filed prior to its grant.

Check either box 1 or 2 below, as appropriate.

1. ☐ For submissions on behalf of an organization (e.g., corporation, partnership, university, government agency, etc.), the undersigned is empowered to act on behalf of the organization.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

2. ☒ The undersigned is attorney or agent of record.

Laurie L. Hill
Signature

November 18, 2002
Date

Laurie L. Hill
Typed or Printed Name

☐ Terminal disclaimer fee of $55.00 under 37 CFR 1.20(d) is included.

☒ Please charge the terminal disclaimer fee of $55.00 to **Deposit Account 03-1952.**

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*Statement under 37 CFR 3.73 (b) is required if terminal disclaimer is signed by the assignee (owner). Form PTO/SB/96 may be used for making this statement. See MPEP § 324.

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Exhibit B

sd-110216

LEXSEE 12 uspq2d 1904

Ex parte David F. Mark, Leo S. Lin, Shi-Da Yu Lu and Alice M. Wang

Appeal No. 88-2811

Application for Patent filed February 7, 1985, Serial No. 06/698,939, which is a continuation-in-part of Serial No. 06/564,224 filed December 20, 1983, now Patent No. 4,518,584; which is a continuation-in-part of Serial No. 06/486,162 filed April 15, 1983, now abandoned; which is a continuation-in-part of Serial No. 06/435,154 filed October 19, 1982. It is also a continuation-in-part of Serial No. 06/698,936 filed February 7, 1985, which is a continuation-in-part of Serial No. 06/670,360 filed November 9, 1984, which is a continuation-in-part of Serial No. 06/661,026 filed October 15, 1984.

Cysteine-Depleted Muteins of Biologically Active Proteins.

Board of Patent Appeals and Interferences

1989 Pat. App. LEXIS 12; 12 U.S.P.Q.2D (BNA) 1904

May 11, 1989, Heard

July 24, 1989, Decided

[*1]

Before Goldstein, Pellman and W. Smith, Examiners-in-Chief.

COUNSEL:

Albert P. Halluin et al. for appellants

Supervisory Patent Examiner - Thomas G. Wiseman

Examiner - Robin Teskin

OPINIONBY:
SMITH

OPINION:

W. Smith, Examiner-in-Chief.

This is an appeal from the final rejection of claims 1 through 5 and 45 through 69. The appeal as to claims 54 and 56 was withdrawn by appellants' counsel at oral argument. n1 Thus, claims 1 through 5, 45 through 53, 55 and 57 through 69 remain for our consideration, which are all of the claims remaining in the application.

n1 It became apparent at oral argument that appellants' invention revolves around the present synthetic muteins retaining the biological activity of the native protein. The method of claims 54 and 56 is not so limited. When this was brought to counsel's attention during oral argument, counsel orally withdrew claims 54 and 56 from appeal.

Claims 1, 45, 54, 55, 56, 57 and 64 are illustrative of the subject matter on appeal and read as follows:

1. A synthetic mutein of a biologically active native protein in which native protein has at least one cysteine residue that is free to form a disulfide link and is nonessential to said biological activity, **[*2]** said mutein having at least one of said cysteine residues substituted by another amino acid and said mutein exhibiting the biological activity of said native protein.

45. A structural gene having a DNA sequence that encodes a synthetic mutein of a biologically active native protein which native protein has at least one cysteine residue that is free to form a disulfide link and is nonessential to said

Exhibit C

biological activity, said mutein having at least one of said cysteine residues substituted by another amino acid and said mutein exhibiting the biological activity of said native protein.

54. A method of preventing a protein having at least one cysteine residue that is free to form a disulfide link from forming said link comprising mutationally altering the protein by deleting the cysteine residue or replacing the cysteine residue with another amino acid.

55. The method of claim 54 wherein the protein is biologically active and the cysteine is not essential to said biological activity.

56. The method of claim 54 wherein the cysteine residue is replaced with serine or threonine.

57. A method for making a gene having a DNA sequence that encodes a synthetic mutein of a biologically [*3] active native protein which native protein has at least one cysteine residue that is free to form a disulfide link and is nonessential to said biological activity, said mutein having at least one of said cysteine residues substituted by another amino acid and said mutein exhibiting the biological activity of said native protein comprising:

(a) hydridizing single-stranded DNA comprising a strand of a structural gene that encodes said protein with a mutant oligonucleotide primer that is complimentary to a region of said strand that includes the codon for said cysteine residue or the antisense triplet paired with said codon, as the case may be, except for a mismatch with said codon or said antisense triplet which mismatch defines a triplet that codes for said other amino acid;

(b) extending the primer with DNA polymerase to form a mutational heteroduplex; and

(c) replicating said mutational heteroduplex.

64. An oligonucleotide for use in making a structural gene, said gene having a DNA sequence that encodes a synthetic mutein of a biologically active native protein which native protein has at least one cysteine residue that is free to form a disulfide link and is nonessential to [*4] said biological activity, and said mutein having at least one of said cysteine residues substituted by another amino acid and said mutein exhibiting the biological activity of said native protein, by oligonucleotide-directed mutagenesis, said oligonucleotide having a nucleotide sequence that is complimentary to a region of the strand of the structural gene that includes the codon for the cysteine residue or the anti-sense triple paired with said codon, as the case may be, except for a mismatch of said codon that defines a triplet that codes for said other amino acid.

The reference relied upon by the examiner is:

Mark et al. (Mark '584) 4,518,584 May 21, 1985

A reference relied upon by the Board is:

Mark et al. (Mark '585) 4,588,585 May 13, 1986

The sole rejection of the claims remaining on appeal is under *35 USC § 112,* first paragraph, as being nonenabled. In support of the rejection, the examiner relies upon a statement of prior art which appears at page 3, lines 22-line 34 of the present specification and at column 1, lines 55-56 of U.S. Patent No. 4,518,584 to Mark et al., one of the present parent applications, which reads as follows:

In this regard Shepard, [*5] H.M., et al, Nature (1981) 294:563-565 describe a mutein of IFN-beta in which the cysteine at position 141 of its amino acid sequence (there are three cysteines in native human IFN-beta at positions 17, 31, and 141, Gene (1980) 10:11-15 and Nature (1980) 285:542-547) is replaced by tyrosine. This mutein was made by bacterial expression of a hybrid gene constructed from a partial IFN-beta cDNA clone having a G—> A transition at nucleotide 485 of the IFN-beta gene. The mutein lacked the biological activity of native IFN-beta leading the authors to conclude that the replaced cysteine was essential to activity.

In addition, the examiner relies upon a statement which appears in an amendment filed in co-pending, commonly assigned Serial No. 06/876,819 which reads as follows:

The review of the newly allowed claims with the inventors in light of the presently available data concerning the claimed species revealed that the seven Cys to Ser substitutions possible within the mature CSF-1 sequence shown in Figure 5 each result in a substantial reduction in the in vitro colony stimulation assay specified in claim 53. Thus, the ser[90]CSF-1 species claimed in claim [*6] 20 (and also in claims 22 and 29) does not meet the requirement specified

by claim 53. Nevertheless, applicants are of the view that the DNA encoding the ser[90]CSF-1 species as well as the other Cys substitution species may have other uses, as experimental probes for example. Accordingly, claim 20 which specifically claims ser[90]CSF-1 has been made independent. For the above described reasons, the ser[90]CSF-1 species has been deleted from claims 22 and 29.

The examiner's rejection is summarized at page 3 of the Examiner's Answer as follows:

Essentially, the position taken in the rejection is that it would require undue further experimentation to construct by recombinant methods (site specific mutagenesis) the innumerable muteins encompassed by the instant claims (claims encompass modification of any protein which comprises a "non-essential" cysteine residue) and to screen the muteins produced for any of those which exhibit biological activity after modification.

The examiner further reasons that it is generally known in the art that cysteine residues facilitate the proper disulfide bonds and consequently the proper folding of a protein. The examiner concludes that it is **[*7]** likely that most of the muteins prepared by appellants' claimed methodology "would be inoperative simply because the removal of the cysteine would disturb proper folding of the molecule, thereby potentially blocking the active site or sites of the resulting mutein." (Examiner's Answer, page 4)

The examiner points out on page 6 of her Answer that the claims on appeal encompass any protein, even those which have not been characterized or cloned and that the mere sequencing of all possible proteins encompassed by the claims on appeal, would entail an undue amount of experimentation.

As set forth on page 7 of the Appeal Brief:

Appellants' position is that given the disclosure of the present invention substituting a nonessential cysteine with a neutral amino acid, the nonessential cysteine residues of any candidate protein could be identified and substituted in ten days employing the methods disclosed in the instant disclosure and the general knowledge of the art at the time the application was filed. Such limited amount of experimentation based on the disclosure in the application and the success shown by three proteins certainly does not constitute undue experimentation.

These arguments **[*8]** are supported by the declaration of co-appellant Alice M. Wang filed under 37 CFR 1.132 on August 10, 1987. In her declaration Ms. Wang sets forth what she terms a reasonable scheme for determining which cysteine residues in a generic biologically active protein would be available for substitution without destroying the biological activity. The declaration sets forth a step-by-step scheme for implementing the claimed invention which parallels the disclosure of the present application.

The examiner sets forth on page 5 of her Answer that Ms. Wang's declaration does not refute the determination that undue experimentation is needed for implementation of the claimed invention because of the "limited successful embodiments shown and the established unpredictability associated with such modifications as to how many such site-specific mutageneses would need to be undergone to obtain even one alternative biologically active mutein."

We have carefully considered the respective positions of the examiner and the appellants and find that we agree with appellants that the claims remaining on appeal are enabled by the present disclosure. The working examples of the present specification set **[*9]** forth experiments which establish that three proteins, IFN-beta, IL-2 and TNF, have nonessential cysteine residues which may be deleted or replaced with the resulting mutein retaining the biological activity of the native protein. When it is considered that the claims remaining on appeal all require that the mutein produced retain the biological activity of the native protein, we consider the disclosure of this application to be enabling. The passages relied upon by the examiner from Mark '584 and copending Serial No. 06/876,819 are merely examples of work which is outside the claims on appeal. The record before us establishes that for a given protein having cysteine residues, one skilled in the art would be able to routinely determine whether deletion or replacement of the cysteine residues would result in a mutein which is within the claims on appeal.

To the extent that the examiner is concerned that undue experimentation would be required to determine other proteins suitable for use in the present invention, we find Ms. Wang's declaration to be persuasive that only routine experimentation would be needed for one skilled in the art to practice the claimed invention for **[*10]** a given protein. The fact that a given protein may not be amenable for use in the present invention in that the cysteine residues are needed for the biological activity of the protein does not militate against a conclusion of enablement. One skilled in the art is clearly enabled to perform such work as needed to determine whether the cysteine residues of a given protein are needed for retention of biological activity.

The examiner's rejection under *35 USC § 112,* first paragraph, is reversed.

NEW GROUND OF REJECTION

Claims 1 through 5, 45 through 53, 55 and 57 through 67 are rejected under *35 USC § 102*(e) as being anticipated by Mark '584 or Mark '585.

The present application lists four co-inventors, Mark, Lin, Lu and Wang. Appellants state on page 1 of the present specification that this application has two lines of parent applications. It is the first line of parent applications, i.e., Serial Nos. 06/564,224, 06/486,162 and 06/435,154 which is of present interest.

This application is stated to be a continuation-in-part of Serial No. 06/564,224 which is a continuation-in-part of Serial No. 06/486,162 which is a continuation-in-part of Serial No. 06/435,154. Each of these **[*11]** parent applications lists only three inventors, Mark, Lin and Lu. Wang, who is a co-inventor of the present application, is not a co-inventor in the parent applications. Mark '584 issued from Serial No. 06/564,224. Mark '585 is a division of Serial No. 06/564,224, and shares common parentage with Mark '584 of Serial Nos. 06/486,162 and 06/435,154.

In order for the present claims to be entitled under *35 USC § 120* to the benefit of the earlier filing date of any of the parent applications, their subject matter must be disclosed in the parent applications in the manner provided by *35 USC § 112,* first paragraph, including the description requirement of this section of the statute. *In re van Langenhoven, 458 F.2d 132, 173 USPQ 426 (CCPA 1972).*

Here, our review leads us to the conclusion that the earliest filing date the present generic claims are entitled to is the December 20, 1983 filing date of parent application Serial No. 06/564,224 since this appears to be the first application in this chain which sets forth a generic description of the synthetic muteins of the present invention. Parent application Serial No. 06/486,162 describes only a synthetic mutein of IFN-beta. The **[*12]** entire original disclosure of Serial No. 06/486,162 describes and is strictly limited to synthetic muteins of IFN-beta except for original claim 20 of that application which was directed to "a nucleotide primer for mutagenesis, comprising an oligonucleotide of about 12 to about 24 bases." The specification of this application contains a corresponding disclosure of this generic nucleotide primer. However, comparing this generic disclosure of a nucleotide primer with that of the present application, i.e., claim 64, it is apparent that claim 20 of this parent application does not provide descriptive support for the broader oligonucleotide disclosed and claimed in this application. Thus, none of the present claims are entitled to the benefit of the earlier filing date of Serial No. 06/486,162, at best, only Serial No. 06/564,224.

Having made this determination, we find that Mark '584 or Mark '585 is available as prior art against the appealed claims under *35 USC § 102*(e) as these patents are by "others" having the effective filing date required by this section of the statute. The effective filing date of these two references, to the extent they disclose synthetic muteins of IFN-beta, **[*13]** is October 19, 1982, the filing date of common parent application Serial No. 06/435,154. They are anticipatory of the claims included in this rejection in that these references describe the IFN-beta synthetic mutein species of the present generic claims. *In re May, 574 F.2d 1082, 1089, 197 USPQ 601, 607 (CCPA 1978).*

Claims 68 and 69 are rejected under *35 USC § 103* as being unpatentable over Mark '584 or Mark '585.

These claims are directed to a therapeutic formulation which comprises an effective amount of the mutein of the present invention and at least one other anti-cancer or anti-viral compound, e.g., gamma-interferon. While Mark '584 and Mark '585 describe such a therapeutic formulation, this description does not appear in common parent Serial No. 06/486,162. This parent application only indicates that the synthetic mutein of IFN-beta is useful for treatment of viral infections, and various types of cancer where interferon therapy is indicated.

However, in view of the disclosed utility of the synthetic mutein of IFN-beta as an anti-cander or anti-viral compound in Serial No. 06/486,162, it would have been prima facie obvious to one of ordinary skill in the art **[*14]** to use the disclosed synthetic muteins of IFN-Beta in combination with other known anti-cancer or anti-viral compounds such as gamma-interferon. *In re Kerkhoven, 626 F.2d 846, 205 USPQ 1069 (CCPA 1980).*

Any request for reconsideration or modification of this decision by the Board of Patent Appeals and Interferences based upon the same record must be filed within one month from the date of the decision (37 CFR 1.197). Should appellants elect to have further prosecution before the examiner in response to the new rejection under 37 CFR 1.196(b) by way of amendment or showing of facts, or both, not previously of record, a shortened statutory period for making such response is hereby set to expire two months from the date of this decision.

37 CFR 1.136(a) does not apply to the times for taking any subsequent action in connection with this appeal.

REVERSED

37 CFR 1.196(b)

PATENT
Docket No. 432722002621

COMBINED DECLARATION AND POWER OF ATTORNEY FOR UTILITY PATENT APPLICATION

AS A BELOW-NAMED INVENTOR, WE HEREBY DECLARE THAT:

Our residence, post office address, and citizenship are as stated below next to our names.

We believe we are the original, first and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled: INHIBITORS OF PROTEASOMAL ACTIVITY FOR STIMULATING BONE AND HAIR GROWTH, the specification of which is attached hereto unless the following box is checked:

☒ was filed on October 20, 1999 as United States Application Serial No. 09/421,545 and was amended on _____ (if applicable).

WE HEREBY STATE THAT I HAVE REVIEWED AND UNDERSTAND THE CONTENTS OF THE ABOVE-IDENTIFIED SPECIFICATION, INCLUDING THE CLAIMS, AS AMENDED BY ANY AMENDMENT REFERRED TO ABOVE.

We acknowledge the duty to disclose information which is material to the patentability as defined in 37 C.F.R. § 1.56.

We hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed:

Application No.	Country	Date of Filing (day/month/year)	Priority Claimed?
			☐Yes ☐No

We hereby claim benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Application Serial No.	Filing Date

We hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, we acknowledge the duty to disclose information which is material to

patentability as defined in 37 C.F.R. § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Serial No.	Filing Date	Status
09/113,947	July 10, 1998	☒Patented ☐Pending ☐Abandoned
09/361,775	July 27, 1999	☐Patented ☒Pending ☐Abandoned

We hereby appoint the following attorneys and agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date

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COPY

PATENT
Docket No. 432722002600

CERTIFICATE OF MAILING BY "FIRST CLASS MAIL"

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

02-02-2001
Date

Julie Brogden

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Gregory R. Mundy, *et al.*

Serial No.: 09/113,947

Filing Date: 10 July 1998, RCE filed on 26 June, 2000

For: INHIBITORS OF PROTEASOMAL ACTIVITY [AND NF-☑B ACTIVITY] AND PRODUCTION FOR STIMULATING BONE GROWTH (AS AMENDED)

Examiner: Ralph Gitomer

Group Art Unit: 1623

DECLARATION OF GREGORY R. MUNDY PURSUANT TO 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Gregory R. Mundy, declare as follows:

1. I am one of the co-inventors of the subject matter claimed in the above-referenced application.

2. Other co-inventors and I have conducted experiments demonstrating that proteasome inhibitors promote osteoblast proliferation, differentiation of osteoblast precursors and bone growth. These experimental results are set forth in the following paragraphs 3-10 and in the attached Tables 1-3.

Exhibit C

3. Osteoblast proliferation, differentiation of osteoblast precursors and bone growth can be shown by histologic observations of calvarial bone cultures, which show increased osteoblast numbers, new bone formation and morphologic maturation of osteoblasts (See detailed description of methods for determining new bone area and osteoblast numbers in Example 2 of the present specification at page 27, line 25 through page 29, line 3).

4. In the experiments described in Example 2 of the present specification, compound 59-0328 serves as a positive control. The two tested proteasome inhibitors are MG-132 and MG-115. As shown in Figure 1 (left column) of the present application as filed, both the positive control compound and the two tested proteasome inhibitors MG-132 and MG-115 promote new bone growth as evidenced by the new bone formation.

5. Promotion of osteoblast proliferation and new bone growth by structurally-unrelated proteasome inhibitors is demonstrated in the Table 1 submitted herewith. In this experiment, the negative control media contain no substance that promotes osteoblast proliferation and new bone growth. BMP2, a substance that is known to promote osteoblast proliferation and new bone growth, serves as a positive control. The four tested structurally-unrelated proteasome inhibitors are lactacystin, proteasome inhibitor 1 (PSI), epoxomicin and eponemycin. The osteoblast numbers treated with the negative control media are 104/0.3 mm bone (Table 1, far right column). The osteoblast numbers treated with the positive control BMP2 are 154/0.3 mm bone. The osteoblast numbers treated with lactacystin (as high as 160/0.3 mm bone), proteasome inhibitor 1 (as high as 179/0.3 mm bone), epoxomicin (as high as 173/0.3 mm bone) are equivalent to or higher than the osteoblast numbers treated with BMP2. The osteoblast numbers treated with eponemycin (as high as 114/0.3 mm bone), although lower than the osteoblast numbers treated with BMP2, is higher than the osteoblast numbers treated with the negative control media. Similarly, the new bone area treated with the negative control media is 3.1 mm^2X10^{-3} (Table 1, second column from the right). The new bone area treated with BMP2 is 5.6 mm^2X10^{-3}. The new bone area treated with lactacystin (as high as 5.9 mm^2X10^{-3}), proteasome inhibitor 1 (as high as 8.2 mm^2X10^{-3}) and epoxomicin (as high as 6.8 mm^2X10^{-3}) is

equivalent to or higher than the new bone area treated with BMP2. The new bone area treated with eponemycin (as high as 3.5 mm^2X10^{-3}), although lower than the new bone area treated with BMP2, is higher than the new bone area treated with the negative control media.

6. Promotion of osteoblast proliferation and new bone growth by another proteasome inhibitor PSI-epoxide is also demonstrated in the Table 2 submitted herewith. The osteoblast numbers treated with the negative control media are 88/0.3 mm bone (Table 2b, middle column). The osteoblast numbers treated with PS1-epoxide are as high as 161/0.3 mm bone (Table 2b, middle column). Similarly, the new bone area treated with the negative control media is 2.8 mm^2X10^{-3} (Table 2a, middle column). The new bone area treated with PS1-epoxide is as high as 5.64 mm^2X10^{-3} (Table 2b, middle column).

7. Osteoblast differentiation can be analyzed by monitoring alkaline phosphatase in the media of these cultures because alkaline phosphatase is an established marker of osteoblast differentiation. Alkaline phosphatase activity in media of neonatal murine calvaria can be measured by the following method. Murine neonatal calvaria are cut and placed into BGJ media with 1 mg/ml bovine serum albumin containing a proteasome inhibitor, *e.g.*, PSI-epoxide, and incubated at 37°C in 5% CO_2 for 24 hours. Four half calvaria are used per treatment group. The media is then changed to fresh media again containing the proteasome inhibitor and incubated for a further 72 hours at which time the media is removed and assessed for alkaline phosphatase activity. Alkaline phosphatase activity is assessed for each calvarial culture by sampling 20 µl of media from each well and reacting it with 80 µl AMP buffer containing p-Nitrophenyl Phosphate, a specific substrate for this enzyme, and incubating for 17 min at 37°C reaction. The reaction is then stopped with 100 µl 0.5N NaOH and the subsequent p-Nitrophenol (yellow color) formation is read at 405 nm and is directly related to the amount of alkaline phosphatase activity present in the cultured media.

8. Promotion of differentiation of osteoblast precursors by eponemycin, PS1 and PS1-epoxide is demonstrated in the Table 3 submitted herewith. In the experiment using eponemycin (Table 3a, middle column), the alkaline phosphatase activity treated with a negative

control media is 0.022 unit. In contrast, the alkaline phosphatase activity treated with eponemycin is as high as 0.075 unit. Similarly, the alkaline phosphatase activity treated with PSI (Table 3b, middle column) and PSI-epoxide (Table 3c, middle column) are also higher than their corresponding negative controls.

9. In the experiments described in the above paragraphs 3-8, it has been observed that the proteasome inhibitors promote osteoblast proliferation, differentiation of osteoblast precursors and new bone growth without any discernible effect on bone resorption.

10. It is known in the art that inhibition of bone resorption does not mean enhancement of bone formation. In fact, reducing bone resorption usually inhibits bone formation because all of bone turnover is slowed. In this aspect, proteasome inhibitors are very different from inhibitors of bone resorption such as bisphosphonates, estrogen, raloxifene or calcitonin, in that the proteasome inhibitors promote osteoblast proliferation, differentiation of osteoblast precursors and new bone growth without any discernible effect on bone resorption.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to by true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Executed at San Antonio, Texas, on January 30, 2001.

Gregory R. Mundy

Gregory R. Mundy

Table 1. Effects of structurally-unrelated proteasome inhibitors and the statin simvastatin at multiple concentrations on explants of neonatal murine calvarial bones cultures for 72 hours.

Treatment	(μM)	New bone area (per $mm^2 \times 10^{-3}$)	Cells (per 0.3 mm bone)
Negative Control Media		3.1 ± 0.2	104 ± 5
BMP2 (40 ng/ml)		5.6 ± 0.4*	154 ± 5*
Lactacystin	0.62	3.2 ± 0.5	105 ± 6
	1.25	4.7 ± 0.7	114 ± 7
	2.5	5.9 ± 0.9*	160 ± 6*
	5	5.4 ± 0.6	157 ± 6*
Proteasome inhibitor-1	0.006	4.3 ± 0.1	116 ± 4
	0.0125	5.5 ± 0.3*	120 ± 6
	0.025	6.0 ± 0.9*	132 ± 10*
	0.05	8.0 ± 0.6*	162 ± 15*
	0.1	8.2 ± 1.2*	179 ± 15*
Epoxomicin	0.00062	2.8 ± 0.3	106 ± 8
	0.00125	2.8 ± 0.4	101 ± 7
	0.0025	4.0 ± 0.4	119 ± 5
	0.005	6.5 ± 0.8*	162 ± 6*
	0.01	6.8 ± 0.5*	173 ± 6*
Eponemycin	0.031	3.1 ± 0.5	98 ± 6
	0.062	2.9 ± 0.3	102 ± 8
	0.125	3.2 ± 0.4	105 ± 7
	0.25	3.4 ± 0.4	96 ± 5
	0.5	3.5 ± 0.8	114 ± 6
Simvastatin	0.062	5.3 ± 1.1	110 ± 9
	0.125	8.2 ± 0.7*	135 ± 4*
	0.25	14.5 ± 1.8*	167 ± 17*
	0.5	14.0 ± 1.1*	190 ± 26*

*Significantly greater than control media $p<0.05$

Table 2

Table 2a. New bone area in the media of murine neonatal calvaria treated with PSI-epoxide for 4 days

Group	New Bone Area ($mm^2 \times 10^{-3}$)	SE
Negative Control	2.80	0.37
PSI –Epoxide 12.5nM	3.46	0.35
PSI –Epoxide 25nM	4.27	0.33*
PSI –Epoxide 50nM	5.64	0.31*
PSI –Epoxide 100nM	5.56	0.20*

Table 2b. Cell number on the surface of murine neonatal calvaria treated with PSI-epoxide for 4 days

Group	Cell Number Per 0.3mm of bone	SE
Negative Control	88	3
PSI –Epoxide 12.5nM	96	4
PSI –Epoxide 25nM	115	8*
PSI –Epoxide 50nM	161	12*
PSI –Epoxide 100nM	151	4*

Table. 3

Table 3a. Relative alkaline phosphatase activity in the media of neonatal calvaria treated with Epoxomicin

Group	Alkaline Phosphatase Activity (OD)	SE
Negative Control	0.022	0.005
Epoxomicin 0.62nM	0.028	0.003
Epoxomicin 1.25nM	0.06	0.001*
Epoxomicin 2.5nM	0.075	0.01*
Epoxomicin 5nM	0.075	0.004*
Epoxomicin 10nM	0.064	0.004*

* - Significantly greater than Control group $p < 0.05$

Table 3b. Relative alkaline phosphatase activity in the media of neonatal calvaria treated with PSI

Group	Alkaline Phosphatase Activity (OD)	SE
Negative Control	0.039	0.005
PSI 12.5nM	0.039	0.004
PSI 25nM	0.072	0.01*
PSI 50nM	0.096	0.01*
PSI 100nM	0.108	0.01*

* - Significantly greater than Control group $p < 0.05$

Table 3c. Relative alkaline phosphatase activity in the media of neonatal calvaria treated with PSI-epoxide

Group	Alkaline Phosphatase Activity (OD)	SE
Negative Control	0.040	0.011
PSI –Epoxide 12.5nM	0.044	0.003
PSI –Epoxide 25nM	0.074	0.006*
PSI –Epoxide 50nM	0.121	0.013*
PSI –Epoxide 100nM	0.107	0.005*

* - Significantly greater than Control group $p < 0.05$

1: Exp Cell Res 1998 Aug 1;242(2):460-9

Related Articles, Links

ELSEVIER SCIENCE FULL-TEXT ARTICLE

The ubiquitin-proteasome system and cellular proliferation and regulation in osteoblastic cells.

Murray EJ, Bentley GV, Grisanti MS, Murray SS.

Geriatric Research, Education and Clinical Center, Department of Veterans Affairs Medical Center, Sepulveda, California, 91343, USA. murrayes@ucla.edu

The 26S proteasome is the macromolecular assembly that mediates ATP- and ubiquitin-dependent extralysosomal intracellular protein degradation in eukaryotes. However, its contribution to the regulation of osteoblast proliferation and hormonal regulation remains poorly defined. Treating osteoblasts with MG-132 or lactacystin (membrane-permeable proteasome inhibitors) attenuates proliferation. Three proteasome activities (peptidylglutamyl-peptide bond hydrolase-, chymotrypsin-, and trypsin-like) were detected in osteoblasts. Catabolic doses of PTH stim-ulated these activities, and cotreatment with PTH and MG-132 blocked stimulation. The proteasome alpha- and beta-subunits, polyubiquitins, and large ubiquitin-protein conjugates were detected by Western blotting. A 90-min treatment with 10 nM PTH had no effect on the amount of proteasome alpha or beta subunit protein, but increased the relative amount of large ubiquitin-protein conjugates by 200%. MG-132 inhibited deubiquitination of large ubiquitin-protein conjugates. The protein kinase A inhibitor SQ22536 blocked much of the PTH-induced stimulation of MCP activities, while dibutyryl cAMP stimulated it, suggesting that protein kinase A-dependent phosphorylation is important in PTH stimulation of proteasome activities. In conclusion, the ubiquitin-proteasome system is essential for osteoblast proliferation under control and PTH-treated conditions. PTH mediates its metabolic effects on the osteoblast, in part, by enhancing ubiquitinylation of protein substrates and stimulating three major proteasome activities by a cAMP-dependent mechanism. Copyright 1998 Academic Press.

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